SYNOPSIS

PhD. in Zoology

Comparative Evaluation of Liposome Encapsulated *Hygrophila auriculata* (Schumach.) Heine Root and Betulin on Wound Healing Activity through *In silico*, *In vitro* and *In vivo* approaches

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Introduction

Hygrophila auriculata (Schumach) Heine (synonym: Asteracantha longifolia Nees, H. schulli (Ham.) MR & SM Almeida, H. spinosa T. Anders, Barleria auriculata Schum and B. longifolia Linn) belongs to family Acanthaceae. It is an erect semi-woody plant found in moistplaces throughout India along the banks of fresh or stagnant water ditches and swampy grounds, mixed with marshy grasses and sedges. Ethnomedicinally the plant has been reported for use in the treatment and prevention of allergy, anemia, arthritis, hypertension, body pain, cancer, renal dysfunction, edema, inflammations, jaundice, liver disorder, leprosy, skin disease, tuberculosis, urogenital disorder and venereal diseases (Dhanalakshmi *et al.,* 2020).

Phytochemicals are the bioactive compounds found in plants that form the basis of many modern-day drugs. They are often used for primary disease prevention or as adjuncts to conventional therapies. Plant-derived chemotherapeutic agents that are cost-effective and efficient are required to regulate the development and progression of wound healing. A suitable balance between low or high levels of reactive oxygen species is essential. Redox imbalance occurs whether the levels of ROS exceed the capacity of endogenous antioxidants to scavenge them, which dysregulates the healing process. Phytochemicals have a potential natural antioxidant that could be more effective than the synthetic ones (Ulbricht and Chao, 2010). Hence in the present study an attempt was made to confirm the presence of biologically active phytochemicals and also the antioxidant potential of roots and leaves of *Hygrophila auriculata*.

Molecular docking is a method used to predict the binding orientation of small molecule drug candidates to their protein targets in order to predict the affinity and activity of the small molecule. In this regard the present study attempts to investigate the lead molecules against wound healing target proteins from *H. auriculata* roots using LCMS analysis by *in silico* docking.

In nanotechnology, vesicular systems, which can be further divided into liposomes, ultra-deformable liposomes, and ethosomes, are composed of amphiphilic molecules that have polar or hydrophilic regions and non-polar or lipophilic regions. Liposomes are spherical vesicles made up of lipid bilayer. The major composition of liposomes is cholesterol and phospholipids. They can encapsulate both hydrophilic and hydrophobic drugs. The epidermal barrier may be weakened or eliminated by wetting the stratum corneum and strengthening hydration, which results in array configuration and structural changes among skin cells and leads to enhanced permeation of liposomes through the extracellular spaces between cells and fusion with skin cells to release the drug (Beltrán *et al*., 2019)

Furthermore, to date, there have been no studies examining the impact of liposome-based drug carrier systems on improving the delivery of *Hygrophila auriculata* root to wound sites, even though these systems are known for their ability to enhance the permeation of drugs through the skin and their localization in the dermis. Hence, the current investigation was conducted using liposome-encapsulated *Hygrophila auriculata* root and its active compound betulin to compare their wound healing efficacy and assess their potential interms of synergistic effects versus the effects of a single active compound.

Skin and soft tissue infections (SSTIs) have high rates of morbidity and mortality associated. Despite the successful treatment of some SSTIs, those affecting the subcutaneous tissue, fascia, or muscle delay the healing process and can lead to life-threatening conditions. In line with this, a significant number of research studies have developed liposomal formulations for skin infections aiming to promote an effective clearance of the pathogen while contributing to skin regeneration and wound healing (Das and Baker, 2016). In the present study, the synthesized liposomes are analysed for antimicrobial properties with both gram positive and gram-negative bacteria.

The wound healing process is very difficult to simulate *in vitro* due to the lack of cell debris and complex interactions among different cell types. However, the *in vitro* ability of a peptide to accelerate the closure of a pseudo-wound by stimulating migration of epithelial cells is indicative of its ability to heal a compromised epithelium. Indeed, cell migration is a rate-limiting event in wound healing, and studying factors that can affect cell migration will help to target therapies for improved wound healing. With this background, the study was formulated with the following objectives:

- To evaluate the phytochemical and antioxidant potential of leaves and roots of *H. auriculata*
- To identify the lead molecules against wound healing target proteins from LCMS of *H. auriculata* roots using *in silico* docking studies
- To characterize liposomes synthesized using the *H. auriculata* roots and the bio active compound betulin
- To evaluate the antimicrobial potential of the liposomes encapsulated *H. auriculata* roots and betulin
- To study the *in vitro* cytotoxicity and wound healing potential of the liposomes encapsulated *H. auriculata* roots and betulin in HACAT– Human Keratinocyte cell lines

- To assess the *in vivo* wound healing activity of liposome encapsulated *H. auriculata* roots and betulin using Swiss albino rat model

Materials and methods

Phase I- Phytochemical and antioxidant studies

Phytochemical analysis and Free radical scavenging

The extracts were subjected to preliminary phytochemical evaluation following the standard color test methods. The radical scavenging activities of the extracts were determined *in vitro* against the battery of radicals namely DPPH, ABTS and reducing power assay.

Estimation of Antioxidant level

The antioxidant level of the ethanol extract of leaves and roots of *H. auriculata* was determined by estimating enzymic and non-enzymic antioxidants. The enzymic antioxidants estimated were catalase, peroxidase, superoxide dismutase, polyphenol oxidase, glutathione-s-transferase and non-enzymic antioxidants estimated were vitamin C or ascorbic acid, flavonoids and tocopherol.

Phase II- *In silico* studies

Evaluation of Ligand Based Docking for wound healing

Ligand optimization of molecules from LC-MS was carried out and the molecules were identified by CAS number from PubChem. Drug likeness of the compounds were analyzed using swiss ADME. Toxicity prediction of selected molecules was assessed using PKCSM tool. The structures of the target proteins Elastase (1HNE), Glycogen synthase kinase-3β (GSK-3β;1Q5K), Gelatinase (1QIB), Collagenase (2Y6I) were obtained from the PDB (Protein Data Bank). The ligands were docked with the target protein using Autodock software.

Plant protein retrieval and its structure validation

UniProt provided the Maturase K (matK) protein sequence obtained from *H. auriculata* in FASTA format. The obtained sequences were physiochemically characterized using the *in silico* ExPASy ProtParam program. A web-based tool, GPMAW [http://www.alphalyse.com/ customer-support/gpmaw-lite-bioinformatics tool/buy-gpmaw/], was used to evaluate the primary Structure of the acquired sequences. Using an online web-based server, SOPMA (Self-Optimized Prediction Method with Alignment), the secondary configuration of the protein sequences was inferred in terms of α helices, β turns, and β sheets proportion expressed in the protein**.** The Three-dimensional Structure of the selected protein was established structurally using x-ray crystallography and also computationally by ERRAT computational tool.

Molecular rigid docking

The obtained protein from the plant is analysed for docking with the wound target proteins using HADDOCK software. All the interface docking regions were analysed. The docking is furthermore facilitated by driving forces that bind the PPIs together.

Phase- III- Liposome synthesis and characterization

Synthesis of liposomes and Spectral studies

The *H. auriculata* and betulin encapsulated liposomes were developed by thin-film hydration method as described by Fathy *et al.,* (2019). The synthesized liposomes were spectrally analysed using XRD, TEM and ZETA potential to determine the morphology and stability of the synthesized liposomes.

Phase IV- Antibacterial activity, *In vitro* cytotoxicity and wound healing activity

Antibacterial activity

The antimicrobial assay adopted the agar well diffusion method against selected bacterial species. The sterile muller hinton agar medium was prepared and swabbed with selected bacterial isolates. The respective samples and standard (ciprofloxacin) were added to separate wells. The bacterial plates were incubated at 37°C for 24 h for five days, and the zone of inhibition was measured.

Cytotoxicity study-MTT assay

Cells were seeded in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allowed the cells to grow for about 24 h. Added appropriate concentrations of the test agent. Incubated the plate for 24h at 37°C in a 5% CO_2 atmosphere. After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{o}C for 4 h to a final concentration of 0.5mg/mL of total volume. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570nm using micro plate reader.

% Cell viability is calculated using below formula:

% cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100

Scratch assay

Seeded cells into 12 well tissue culture plate at a density of 0.25 million cells per well, until they reach ~80-100% confluence as a monolayer for the incubation period of 24h.

Gently and slowly scratched the monolayer with a new pipette tip across the centre of the well. After scratching, gently washed the well twice with medium to remove the detached cells. Treated the cells with desired concentrations of given compound prepared in media and incubated at 37^{o}C with 5% CO_2 in the incubator. Grew cells for additional 48h (or the time required if different cells are used). Captured the cell images at different time intervals (ex: 0, 24h and 48h). The gap distance was quantitatively evaluated using software such as ImageJ.

% Wound Healing Scored= (Initial Area-Final area)/Initial area *100

The direct microscopic observations of images of test compound, standard control was carried out after 0 h, 24 h and 48 h of incubation.

***In vivo* studies**

Wound healing activity - Excision model

The animals were divided into four groups as given below, each group containing of four animals.

Group I served as excision wounded animals without treatment,

Group II and III served as wounded animals treated with liposome encapsulated *H. auriculata* and liposome encapsulated Betulin ointment applied topically for 16 days

Group IV served as wounded animals treated with Standard Drug povidone ointment applied topically for 16 days.

The rats were anaesthetized using ketamine prior to creation of wounds. The dorsal fur of the animals was shaved with an electric clipper and wound was created on dorsal thoracic region. The full thickness of the excision wound was created around 2.5cm in length, 0.2cm in depth by using surgical blade, pointed scissors and toothed forceps. The wound was left open. All the rats were given regular dressing changes and kept for observation. The wound closure rate was assessed by tracing the wounds on days 0, 4, 8, 12 and 16.

The percentage of wound contraction was calculated by the following formula,

Percentage wound contraction = Healed Area / Total Area X 100

After the experimental period, the animals were sacrificed by cardiac puncture and the blood and tissue samples were collected for analysing biomarker parameters such as IL– 6, procalcitonin and C-reactive protein. They were also analysed for haematological parameters such as macrophages, neutrophils, T-lymphocytes, platelets and erythrocyte sedimentation rate. The collected tissues were subjected to histopathological analysis.

Results

Preliminary phytochemical and antioxidant studies

- The findings revealed the existence of alkaloids, terpenoids, sterols, carbohydrates,

saponins, and flavonoids, with the highest intensity observed in the ethanol extract in both the leaves and roots.

- In the DPPH assay, the extent of DPPH scavenging by all the extracts was significant, where the stable radical was effectively reduced to the yellow-coloured compound di phenylpicryl hydrazine. Among the four solvents used, the ethanol extract of leaves and roots of *H. auriculata* had the highest free radical scavenging capacity with an IC_{50} value of 75 µg/ml and 67 µg/ml followed by ethyl acetate extract.
- The extent of ABTS radical scavenging was higher in the ethanol extract in the leaves and roots of *H. auriculata* followed by the ethyl acetate and aqueous extracts. The IC_{50} value of ethanol extract of the leaves and roots of *H. auriculata* were 77 µg/ml and 70 µg/ml. The chloroform extract showed the lowest effect.
- The reducing capacity of a compound serves as a significant indicator of its antioxidant capacity. The presence of reductants in all the extracts resulted in the reduction of ferric cyanide complex to ferrous form with the development of Perl's Prussian blue. This can be monitored at an absorbance of 700 nm and found that the ethanol extract of the leaves and roots of *H. auriculata* showed highest reduction.
- The results of the free radical scavenging assays showed that the ethanol extract exhibited highest free radical scavenging activity in cell free systems.
- The levels of enzymic and non-enzymic antioxidants showed the following results:
  SOD and POD in leaves and roots were found to be 11.5 ± 0.7, 13.3 ± 0.5 and 6.11 ± 7.33, 9.34 ± 2.11 U/g tissue respectively. In this study, the catalase level of leaves and roots was found to be 1.03 ± 0.5 and 4.53 ± 0.9 U/g tissue and polyphenol oxidase was found to be 0.1 ± 0.01 and 0.4 ± 0.01 µg/g in leaves and roots respectively. The level of GST was 1.8 ± 0.1 and 2.3 ± 0.5 µg/g in leaves and roots respectively.
- Of the three non- enzymic antioxidants analyzed, the level of flavonoids was found to be 1.36 ± 0.53 and 2.40 ± 1.23 µg/g. The level of α-tocopherol was found to be 26.5 ± 1.97and 32.3 ± 0.18 µg/g and Vitamin – C were found to be 1.88 ± 0.12 and 2.93 ± 0.02 µg/g.
- Among the leaves and roots of *H. auriculata*, the ethanol extract of the roots of *H. auriculata* was found have maximum amount of enzymic and non-enzymic antioxidants.

LCMS analysis

- The bioactive compounds of the *H. auriculata* root were qualitatively characterized by LCMS analysis in both positive (+) and negative (-) modes. The identified compounds

belong to secondary metabolites like terpenoids, alkaloids, aliphatic compounds, and phenolics.

- The resulting LC-MS from *H. auriculata* root has totally 15 molecules. The spectrum profile of the LC-MS data of *H. auriculata* was verified with the PUBCHEM database. These compounds were further chosen for *in silico* docking analysis with the target proteins responsible for the pathogenesis of wound.

Drug likeness and ADMET properties

- The drug likeness properties of the 15 compounds were assessed by using Swiss ADME. Out of 14 molecules, only 10 molecules were found to satisfy druglike properties based on Lipinski's rule of five. In the present study the compounds such as Caffeic anhydride, Quercetin, Myristic acid, Palmitic acid, Coumaroyl quinic acid, Epiafzelechin, Kaempferol-7-O-Glucoside, Betulin, Linoleic acid and Chlorogenic acid were found to be highly druglike.
- These selected compounds were further evaluated for their drug-like behavior through analysis of pharmacokinetic parameters required for absorption, distribution, metabolism, excretion and toxicity (ADMET) by using PKCSM. Overall, all the compounds showed significant ADMET properties that enable every compound to be defined as druglike and non-toxic
- Following the physicochemical properties, the bioactive scores were evaluated. The bioactivity analysis revealed that all the compounds were highly bioactive except myristic acid, 1,3-Dicaffeoylquinic acid, and Apigenin-6-C-glucoside 8C-arabinoside.

Molecular docking studies

Ligand-protein docking

- Totally 40 *in silico* docking analyses were performed. The entire docked complex was visualized using discovery studio. Among the docked compounds betulin (-9.65 kcal/mol), chlorogenic acid (9.12 kcal/mol), Kaempferol-7-O-Glucoside (-8.19 kcal/mol), Linoleic acid (-7.96 kcal/mol) and Epiafzelechin (-6.62 kcal/mol) showed the highest binding affinity towards the target proteins.

Maturase K and its structure validation (Protein-protein docking study)

- Despite ligand protein docking, protein-protein interaction also plays an important role in many cellular pathways and key role in drug targeting. Hence the protein Maturase K which is the only protein, from *H. auriculata*, available in Uniprot database had been chosen for docking studies. The sequence of Maturase K was retrieved from Uniprot

database and further analysed for its structure and stability.

- The Maturase K had 296 amino acids which denotes that the protein is stable due to the high quantity of amino acids. Since the result showed a 10.37 pH value for theoretical PI, it indicates a highly basic protein. The instability index predicts protein stability, with a value less than 40 indicating that the protein Maturase K is stable. The presence of 94.46 % aliphatic amino acids signify that Maturase K protein is highly aliphatic. The negative GRAVY score refers to the hydrophilic nature of the protein.
- Maturase K protein had the highest percentage of aliphatic amino acids, such as Alanine, valine, leucine, isoleucine, and methionine. In contrast, it contained the lowest percentage of cysteine. The acidic group includes aspartic acid and glutamic acid, whereas the primary group includes arginine, histidine, and lysine. The protein also contained the aromatic amino acids phenylalanine, tryptophan, and tyrosine.
- The percentage score of amino acid distribution in secondary structure infers that the alpha helix is dominated over other secondary structures, followed by the random coil, extended strand, and beta-turn. Maturase K had the highest percentage of α-helices (39.53 percent) in secondary structure analysis compared to others indicating that the protein is thermally resistant based on their intrinsic stability
- Maturase K was shown to have good quality characteristics with the ERRAT score of 91.00. This computer investigation also confirms the crystallographic tertiary structure of the protein. Here the QMEAN of -1.89, which is near 0, showed that the quality of the model is satisfactory.
- The Ramachandran plot favors the first quadrant of amino acids, representing around 73.9 percent of the total, showing that the protein is stable.
- With a protein-protein interaction (PPI) enrichment p-value of $<1.0e^{-16}$, an average local clustering coefficient of 1 was predicted. This suggests that the proteins of interest interact with one another more than a random set of proteins of similar size would.
- The Z score obtained for Maturase K with the proteins GSK, Collagenase, Elastase, and Gelatinase were -1.4, -2.7, -2.2, and -2.1 respectively. The highest negative value was shown by Collagenase, followed by GSK, Elastase, and Gelatinase.
- Since betulin exhibited significant docking interaction with wound target proteins when compared to the Maturase K, it could be substantiated that betulin is an efficient drug candidate for wound healing and hence for further studies commercial Betulin had been used.
- Overall, in the ligand-protein docking, the compound betulin showed the highest binding affinity with the wound pathogenic protein. The ADMET properties also showed

significant results for betulin. Hence the presence of terpenoid, betulin was also assessed in comparison with the *H. auriculata* root extract for the wound healing studies.

Liposome synthesis and its characterization

- The liposomes used in the present study had the encapsulation efficiency of 79.2% and 75% EEHA and betulin respectively.
- The diffraction pattern of the liposomes synthesized using *H. auriculata* root extract indicated the existence of Face Centered Cubic structure (FCC). The high intensity of these diffraction peaks indicated the crystalline nature of the sample. The mean size was found to be 30.98 nm which is almost similar to the average size obtained using TEM analysis.
- From the TEM analysis of the liposomes synthesized using *H. auriculata* root extract and with the compound betulin it was observed that the size of the nanoparticles ranges from 22.655 nm to 37.986 nm and spherical in shape.
- The zeta potential of the liposomes synthesized using *H. auriculata* root extract was -23.9 mV and for liposomes synthesized using betulin, it was seen to be -51.7 mV.

Antibacterial activity

- In the present study, the antimicrobial efficacy of the active compound betulin from *H. auriculata* and the ethanol extract of *H. auriculata* were assessed before and after the liposome encapsulation. The liposome-encapsulated *H. auriculata* extract showed the highest inhibition of all the bacterial species compared to all other samples. The values of the zone of inhibition ranged from 12.3 ± 0.57 mm to 22.2 ± 2.3 mm which denotes a very better effect in comparison with control, which is from 18.6 ± 1.15 mm to 27.6 ± 3.2 mm.

MTT assay

- In MTT assay, HACAT cells showed that sample concentration is indirectly proportional to the cell viability. When the sample concentration increases, the viability was found to decrease. Liposome concentrations of 12.5–100 μg/mL tended to have higher cell viability than blank and positive treatments. This indicated that liposome showed an effect on cell viability and was non-toxic to HACAT cells at concentrations less than 100 μg/mL.
- The IC_{50} value of LHA and LB (LHA- Liposome of *H. auriculata* LB- Liposome of betulin) was found to be 120.25 μg/ml and 170.09 μg/ml. Liposomes synthesized using *H. auriculata* root showed a better cell viability as compared to the liposomes synthesized using betulin.

Scratch assay

- The results of Wound healing assay suggested that both the liposomes were effective in healing potency on Human Skin Keratinocytes (HACAT) which decreased the area of wound on incubation dependent manner.
- There was a significant increase in the percentage of cell migration with time in the HACAT cells that were treated with the Liposomes synthesized using *H. auriculata* root when compared with the Liposomes synthesized using betulin. The cells treated with Liposomes synthesized using *H. auriculata* root extract migrated to a significantly greater extent when compared with the 0h of the treated cells.
- Collagen1 was expressed by 78.35% of the HACAT cells treated with liposomes, much similar to the percentage of cells treated with the standard control that expressed collagen 1, for 84.89%, suggesting the induction of collagen 1 expression in HACAT cells by the liposomes.

Excision wound healing activity

- Treatment with LHA 10% (w/w) liposome ointment showed significant wound contraction ($p < 0.01$) on day 8 and on other post wounding days ($p < 0.01$ and $p<0.05$) as compared to standard ointment. Similarly, treatment with LB ointment showed significant wound contraction on all post wounding days, on day 12 ($p < 0.01$), and on the rest of the post-wounding days ($p < 0.01$) as compared to standard ointment. Here the synergistic effect of liposome encapsulated *H. auriculata* ointment showed significant healing than the liposome encapsulated betulin.
- The histology of the healed wound after being treated with liposome showed normal appearing of epidermis and dermis. The dermis showed increased number of blood vessel and inflammatory infiltrate. The LHA (Liposome encapsulated *H. auriculata* roots) treated wound showed normal appearing epidermis and dermis with normal adnexae. Similarly, positive control povidone treated show normal appearing epidermis and dermis with increased blood vessel.
- The levels of IL-6 and CRP were found to be lower in excision wounded treated rats when compared to normal untreated rats. On treatment with LHA liposome ointment, the levels of IL-6 and CRP were significantly reduced as compared to the LB and positive control. Similar results were recorded for the procalcitonin.
- The hematological parameters such as macrophages, T Lymphocytes, platelets and ESR showed a better decline in the values as compared to the untreated which is nearer to the standard.

Summary and conclusion

- The study was conducted in four different phases. In phase I, phytochemical studies and free radical scavenging activity of different extracts of *H. auriculata* leaves and roots was analysed followed by the estimation of enzymic and non-enzymic antioxidant levels.
- The maximum intensity of phytochemicals was recorded in the ethanol extract of roots of *H. auriculata*
- Free radial scavenging assays established that ethanol extract of *H. auriculata* exhibited outstanding scavenging effects on DPPH, ABTS and FRAP
- The antioxidant levels of the EEHA were determined by estimating enzymic and non-enzymic antioxidants in which the maximum amount of antioxidants were observed in the roots and found to be significant
- In phase II, the *in-silico* studies were carried out and the lead compounds against the wound targets were identified from the secondary active constituents of *H. auriculata* roots using the ligand-based docking.
- Eight complexes showed higher docking score among which betulin represented the highest. Hence betulin was chosen for further analyses of *in vitro* and *in vivo* studies to examine the comparative efficiency between a single bioactive compound and the whole root extract.
- In phase III, the liposome was synthesized and characterized using the EEHA and also with the active compound betulin.
- The liposomes synthesized showed a good spectral property with FTIR and XRD and a better stability with TEM and ZETA potential
- Antimicrobial study was done with the synthesized liposomes where the LHA showed significant zone of inhibition.
- In the final phase of the study, the wound healing activities were done *in vitro* and *in vivo* using the cell lines and the Swiss albino rat model.
- In the MTT assay, the IC_{50} of LB and LHA was found to be 120.25 µg/ml and 170.09 µg/ml respectively showing less toxicity
- In the scratch assay, there was a significant increase in the percentage of cell migration with the Liposome synthesized using *H. auriculata* root when compared with the Liposomes synthesized using betulin. HACAT cells treated with the liposome for 48 hshowed a significant increase in the collagen 1 expression and were similar to the positive control
- In the *in vivo* study, the results exhibited a better wound contraction in liposome of

h. auriculata root treated wounds when compared to the liposome of betulin. In the day 16, LHA showed the complete healing of wound as compared to the standard

- The elevated levels of the biomarkers such as IL 6, CRP and procalcitonin delays the wound healing. In the present study, the significant decrease of the biomarkers indicates formation of keratinocytes thereby quickening the healing process.
- Thus, the present investigation scientifically validates wound healing properties of *H. auriculata* root with liposome encapsulation and strengthens it as a potential candidate for the development of safer drugs for wound healing.
- Altogether this study throws light on the synergistic effect of the *H. auriculata* root encapsulated liposome as a potential wound healing agent owing to its remarkable antioxidant, antimicrobial and wound healing properties.
- Overall, betulin also has potential *in vitro* and *in vivo* wound healing effects but as the traditional knowledge implies the synergistic effect plays a vital role in the efficiency when compared to that of the effect of single bioactive compound
- Above all, there is a need for much targeted research, based on specific hypothesis, to maximize the success rate of future investigations

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